9 (±) HODE ELISA

Product NWK-9HOD01
For Research Use Only

Assay system for measurement of 9 (±) Hydroxyoctadecadienoic Acid (HODE), a metabolite of linoleic acid and marker of lipid peroxidation.

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Introduction:

Linoleic acid, the predominant polyunsaturated fatty acid (PUFA) in the human diet, can be metabolized by cyclooxygenase, lipoxygenase and P450 enzymes. The hydroxyoctadecadienoic acid (HODE) derivatives of linoleic acid, 9(R)-HODE, 9(S)-HODE and 13(S)-HODE, are the most widely distributed of the known linoleic acid metabolites. These compounds exhibit interesting biologic activities, including the regulation of platelet function, maintenance of vascular thromboresistance and transduction of the cellular responses to certain growth factors. HODE derivatives may also influence certain pathological states including, psoriasis, the development of atherosclerosis and the development of cancer.

Intended Use:

This kit is intended for the quantification of total 9-HODE, which includes both 9(S)-HODE and 9(R)-HODE, in biological samples.

Test Principle:

This kit is a competitive enzyme-linked immuno-assay (ELISA). Briefly, the 9-HODE present in the samples or standards competes with 9(±)-HODE conjugated to horseradish peroxidase [9(±)-HODE-HRP] for binding to an antibody specific for 9(±)-HODE that is precoated on a microplate. The peroxidase activity of 9(±)-HODE-HRP results in color development when a substrate is added. The intensity of the color is proportional to the amount of 9(±)-HODE-HRP bound and is inversely proportional to the amount of unconjugated 9-HODE present in the samples or standards.

General Specifications:

Format:	96 well competitive ELISA
Number of tests:	Triplicate = 24 Duplicate= 40
Specificity:	9(±)-hydroxyoctadecadienoic acid
Sensitivity:	0.1 ng/mL
Effective Range:	0.1ng/mL - 500 ng/mL

Kit Contents

Microwells precoated with anti-9(±)-HODE:	1 X 96 wells
9(±)-HODE Standard (50 µg/mL in ethanol)	1 X 20 µL
Dilution Buffer (5X)	1 X 25 mL
5X Wash Buffer:	1 X 50 mL
TMB Substrate:	1 X 25 mL
9(±)-HODE-HRP Conjugate (40X):	1 X 320 µL
Reagent Trough:	2 Each

Required Materials Not Provided:

Adjustable pipettes with a range of 10 µL to 1,000 µL with disposable tips.

Reagents for extraction of samples. Suggested extraction requires ethyl acetate, hydrochloric acid, methanol, and a means for drying the sample, such as a centrifugal evaporator, nitrogen or argon gas.

Plastic-ware for preparation of reagents.

Deionized water.

3N sulfuric acid.

Required Instrumentation:

Microtiter plate reader with 450 nm capability.

Warnings, Limitations, Precautions:

Individual components may be harmful if swallowed, inhaled or absorbed through the skin. Contact should be minimized through the use of gloves and standard good laboratory practices. If contact with skin or eyes occurs, rinse the site immediately with water and consult a physician.

NOTE: 9(±)-HODE may bind to glassware. Therefore, the use of plastic-ware (polypropylene) or silanized glassware is recommended for all procedures involving the standards, enzyme conjugate, and samples containing 9-HODE.

Storage Instructions:

Store all components at 4°C until immediately before use. Do not freeze.

Assay Preparation

1. Determine the number of wells required to assay standards, samples and controls for the appropriate replicate.

2. Create an assay template showing positioning of standards, controls and samples. Include blank wells also.

Assay Preparation (Continued):

3. Bring all samples and reagents to room temperature before use. To avoid condensation, do not open foil pouches containing the microtiter strips until after they have reached room temperature.

4. Next remove the required number of strips and place in the frame supplied. Return unused wells to the storage bag, seal and store at 2-8ºC.

Reagent Preparation:

The following instructions are based on the user using the entire kit at one time. If portions of the kit are to be used at a later time, smaller quantities may be prepared saving the remaining stock for later use.

TMB Substrate is supplied ready to use.

Dilution Buffer

Add the contents of the 5X Dilution Buffer (25 mL) to 100 mL deionized water. Label as **Working Dilution Buffer.**

5X Wash Buffer

Add the contents of the 5X Wash Buffer (50 mL) to 200 mL deionized H_2O, mix well and label as **Working Wash Buffer.**

40X 9(±)-HODE-HRP-Enzyme Conjugate

Add 300 µL 9(±)-HODE-HRP Conjugate to 11.7 mL Dilution Buffer. Label as **Diluted HRP-Conjugate.**

Standard Preparation:

Standard Supplied: 9(±)-HODE: 1 X 20 µL at 50 µg/mL in ETOH.
Add 20 µL of the 50 µg/mL standard supplied to 980 µL **Working Dilution Buffer.** Label as **1000 ng/mL Standard.**

Standard 7 (S_7): Add 400 µL of the 1000 ng/mL Standard to 400 µL of Working Dilution Buffer. *Label as* ***500 ng/mL.***

Standard 6 (S_6): Add 200 µL of S_7 to 800 µL Working Dilution Buffer and vortex. *Label as* ***100 ng/mL.***

Standard 5 (S_5): Add 100 µL of S_6 to 900 µL Working Dilution Buffer and vortex. *Label as* ***10 ng/mL.***

Standard 4 (S_4): Add 400 µL of S_5 to 400 µL Working Dilution Buffer and *vortex. Label as* ***5 ng/mL.***

Standard 3 (S_3): Add 200 µL of S_4 to 800 µL Working Dilution Buffer and vortex. *Label as* ***1 ng/mL.***

Standard Preparation (continued):
Standard 2 (S_2): Add 400 µL of S_3 to 400 µL Working Dilution Buffer and vortex. *Label as* ***0.5 ng/mL***.

Standard 1 (S_1): Add 200 µL of S_2 to 800 µL Working Dilution Buffer and vortex. *Label as* ***0.1 ng/mL.***

Standard 0 (S_0): Add 400 µL Working Dilution Buffer only to this tube. Label as **0 ng/mL.**

Sample Handling/Preparation
Note: In order to determine extraction efficiency for a specific sample type we recommend that users measure the 9(±)-HODE concentration of a parallel sample (i.e. a biological sample to which a known amount of 9(±)-HODE is added prior to extraction).

Determination of Free and Esterified 9(±)-HODE in Plasma & Other Fluids
1. Prepare Folch solution (2:1 CHCl3/MeOH) with Butylated hydroxytoluene (5 mg BHT/100 mL) and Triphenyl phosphine (50 mg TPP/100 mL).

2. Add 20 mL Folch solution to a 50 mL conical tube and place on ice.

3. Add 1 mL plasma or other fluid then shake or vortex well for 1 minute.

4. Add ice cold 0.43% MgCl2 (10 mL) and shake or vortex well for 1 min.

5. Centrifuge for 2-3 min.

6. Aspirate off the top layer (MgCl2/MeOH) and transfer the organic layer to another 50 mL tube, being careful not to transfer any protein layer that may be present.

7. Dry the organic layer under N2.

8. Add 0.5 to 2 mL MeOH (depending on the amount of lipid present) containing BHT (5 mg/100 mL) and an equal volume of 15% KOH. Swirl after each addition.

9. Allow sample to stand at 37 °C for 30 minutes.

10. Adjust pH to 3 with 1 N HCl using approximately 2.5 times the volume of 15% KOH that was added.

10. Dilute with pH 3 water so that the volume of MeOH added is ≤ 5% of the total volume. The sample is now ready for solid phase extraction as described below.

Sample Handling/Preparation (continued)

Determination of Free and Esterified 9(±)-HODE in Tissue Samples

1. Prepare Folch solution (2:1 CHCl3/MeOH) with BHT (5 mg/100 mL).

2. Add 20 mL Folch solution to a 40 mL flat bottom tube and place on ice.

3. Weigh 0.5 to 1 gram tissue and add to tube on ice. Shake or vortex well for 1 min.

4. Homogenize with blade homogenizer

5. Allow to stand under N2 in a sealed tube for one hour at room temp, vortexing occasionally.

6. Add 4 mL 0.9% NaCl.

7. Vortex vigorously and centrifuge for 2-3 min.

8. Discard the upper layer (MeOH/saline).

9. Remove the lower phase to 100 mL Rota-VAP flask or 50 mL conical tube, avoiding the protein layer.

10. Evaporate and add 2-4 mL MeOH with 5 mg BHT/100 mL and an equal volume of 15% KOH.

11. Place in a water bath at 37-40°C for 30 minutes.

12. Adjust pH to 3 with 1 N HCl using approximately 2.5 times the volume of 15% KOH that was added.

13. Dilute to 40 – 80 mL with pH 3 water so the MeOH is ≤ 5% of the total volume. The sample is now ready for solid phase extraction as described below.

Extraction of 9(±)-HODE from serum, plasma or tissue culture media:

1. Acidify to pH 3 or 4 with concentrated HCl.

2. Extract with 3x sample volume of water saturated ethyl acetate, centrifuge at low speed or allow to stand until phases separate.

3. Remove organic (upper) phase and transfer to new container, being careful not to contaminate with aqueous phase.

4. Repeat steps 2 and 3, combining organic phase with that from first extraction.

5. Dry down completely under nitrogen or in a centrifugal evaporator.

6. Bring samples up in 25 µL methanol, then add 975 µL Working Dilution Buffer. (If solubility is a problem, it may be necessary to increase the pH.)

NOTE: This ELISA assay is sensitive to differences in pH among samples and/or standards. Therefore, it is critical to ensure that all samples and standards are adjusted to the same pH prior to running the assay.

Assay Protocol:

1. Remove plate from foil pouch. Place **100 µL of standards** and **diluted samples** into the appropriate wells. Reserve 2 wells to be used as blanks to which no Diluted HRP-Conjugate or substrate is to be added.

2. Add **100 µL of the Diluted HRP CONJUGATE** to each well. Allow plate to stand at room temperature, covered, for 2 hours.

3. Invert the plate and empty the contents. Pat dry upside-down on a lint free paper towel.

4. Wash all wells by adding 400 µL Working Wash Buffer to each well, wait 2 minutes, then invert the plate to empty the contents. Pat dry upside-down on a lint free paper towel. Repeat 2 more times for 3 washes.

5. Add 200 µl SUBSTRATE to each well and allow to incubate, covered at room temperature, for 45-60 minutes. This allows for color development to occur.

6. Add 50 µL of 3 N sulfuric acid to each well and then read the plate at 450 nm.

Data Analysis

1. Average the reagent blank absorbance values and subtract this average from each well. Most modern microplate readers are capable of doing this automatically.

2. Average standard replicates (S_1 through S_7) and divide by the average obtained for S_0 (Zero Bound or B_0) to express data as a percent of B_0.

3. Graph $\%B_0$ values (y-axis-linear) vs. standard concentration (x-axis-logarithmic) to obtain a standard curve. Figure 1 shows a typical curve obtained when plotting concentration vs. percent bound in this fashion.

Figure 1.

4. Average the replicates of each unknown and divide by the average S_0 (B_0) value to express data in terms of $\%B_0$, then determine corresponding concentration using the standard curve. Remember to account for any dilution factor incurred by samples prior to assay.

Performance Details:

9(±)-HODE Crossreactivity:

9(±)-HODE	100.0%
9(S)-HODE	100.0%
9(R)-HODE	100.0%
13-oxo-octadecadienoic acid	2.4%
9-oxo-octadecadienoic acid	1.2%
13(S)-hydroxyoctadecadienoic acid	1.2%
13(R)-hydroxyoctadecadienoic acid	1.2%
11(S)-HETE	0.0%
15(S)-HETE	0.0%
Linoleic acid	0.0%

REFERENCES

1. Spindler, S.A., Clark, K.S., Callawaert, D.M., Reddy, R.G., *Biochem. Biophys. Res. Comm.,* **218** : 187-191 (1996)